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Lipids of Normal Brain

By A. C. JOHNSON (National Research Council Fellow), A. R. McNABB (National Research Council Fellow) AND R. J. ROSSITER, *Department of Biochemistry, University of Western Ontario, London, Canada*

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In most of the early studies on the distribution of the lipids in the brain large samples of tissue were used, and the various fractions isolated, more or less quantitatively, by the classical differential solubility techniques. Extensive reviews of this work have been given by Page (1937) and Bloor (1943).

To investigate experimentally degenerative conditions in the nervous system, methods must be available which require only small samples of tissue. In recent years many such micro methods have been reported. In the experiments described in this paper the micro methods at present in use in this laboratory have been applied to normal brain tissue.

METHODS

Reagents. All reagents used were of A.R. grade, and all solvents were freshly redistilled on the day of use.

Extraction of lipids

The brain was dissected out as soon as possible after the animal had been killed. A small sample of tissue (1–2 g.) was rapidly weighed, and extracted by a modification of the

method of Bloor (1929). It was ground in a mortar with a small quantity of acid-washed, ether-extracted sand, and successively extracted with 50 ml. ethanol (2 portions), 50 ml. 1:1 ethanol-ether mixture (8 portions) and 50 ml. ether (1 portion). Each extract was filtered in turn through an ether-extracted Whatman no. 2 filter paper into a 500 ml. volumetric flask, and the contents of the flask finally made up to volume with ether. By this procedure, in which the proportion of solvent to tissue was greater than that usually adopted, the lipids were completely extracted from brain tissue. To verify this, the insoluble residue was extracted for 6 hr. with ether in a continuous extraction apparatus, followed by a further 6 hr. with methanol. The combined extract was found to contain neither cerebroside nor cholesterol and only negligible quantities of phospholipid. When the same procedure was applied to liver tissue, however, it was found that a considerable phospholipid fraction was not extracted.

Three separate 100 ml. samples of the ether-ethanol extract were placed in a 60° water bath, and evaporated just to dryness under reduced pressure and in an atmosphere of N₂. The residue was extracted successively with eight 2 ml. portions of light petroleum (b.p. 40–60°) and the light petroleum evaporated down to 1 ml. on a 60° water bath. The phospholipids were precipitated by adding 30 ml.

acetone and 20 drops of a saturated solution of $MgCl_2$ in ethanol. After centrifuging, the clear supernatant liquid was decanted into a 50 ml. volumetric flask. The ppt. was washed with 10 ml. acetone and the acetone washings were added to the 50 ml. volumetric flask which was then made up to volume with acetone. The ppt. was dissolved in 5 ml. of a 1:1 methanol-ether mixture, and transferred quantitatively to a 25 ml. volumetric flask which was made up to volume with 1:1 methanol-ether mixture.

All analyses were done in triplicate, the sample for the cerebroside estimation being taken from the original ethanol-ether extract, those for the cholesterol from each of the three 50 ml. volumetric flasks containing the acetone-soluble material, and those for the phospholipids from each of the three 25 ml. volumetric flasks containing the methanol-ether solution.

Free and total cholesterol were estimated in 2 ml. of the acetone solution by a modification of the method of Schoenheimer & Sperry (1934), incorporating improvements suggested by Sperry & Brand (1943) and Sobel & Mayer (1945). The colour was read in a Coleman Universal spectrophotometer at 620 $m\mu$.

Cerebrosides were estimated by the method of Brand & Sperry (1941) in which the galactose liberated on hydrolysis is estimated by the ceric sulphate titration procedure of Miller & Van Slyke (1936). Six 25 ml. samples were taken from the original ethanol-ether extract and, of these, three were hydrolyzed, and three treated in exactly the same way, except that the hydrolysis was omitted. Eriogreen proved to be a more satisfactory indicator than either setopaline C or the *o*-phenanthroline ferrous complex suggested by Macy (1942). It was found that more reproducible results could be obtained if the unhydrolyzed as well as the hydrolyzed sample was neutralized to chlorophenol red before the clarification procedure.

Phospholipids. The procedure adopted for the determination of the various phospholipids was the same as that described by Hack (1947). It depends on the observation that the monoaminophospholipids, lecithin and kephalin, are completely hydrolyzed by N -KOH in 18 hr. at 37° whereas, under these conditions, there is no hydrolysis of sphingomyelin. The total acid-soluble P in the hydrolysis mixture is a measure of the lecithin and kephalin present (Schmidt, Benotti, Hershman & Thannhauser, 1946) and the choline a measure of the lecithin (Hack, 1947). The difference between the total P and the total acid-soluble P in the hydrolysate is a measure of the sphingomyelin, and the difference between the molecular equivalents of monoaminophospholipid and choline gives the molecular equivalents of kephalin.

For the estimation of total lipid P, 1 ml. was taken from the 25 ml. volumetric flask containing the methanol-ether solution of the phospholipids, and the total P was determined by the method of Fiske & Subbarow (1925). The phospholipid was calculated on the basis of

$$\text{total phospholipid} = \text{total lipid P} \times 25.$$

The monoaminophospholipid was estimated in the remaining 24 ml. of the methanol-ether solution which was evaporated under reduced pressure at 60° to a volume of 0.2 ml. After the addition of 5 ml. N -KOH, the tube was stoppered and shaken at 37° for 18 hr. To 0.5 ml. of this solution, pipetted into a centrifuge tube, there were added 0.1 ml. 5*N*-HCl and 3 ml. 10% (w/v) trichloroacetic acid.

The centrifuge tube was stoppered, shaken, and kept for 1–2 hr. at room temperature. After centrifuging, the solution was filtered through a Whatman no. 44 filter paper, and the total P estimated in 2 ml. of the trichloroacetic acid filtrate (representing 1.28 ml. of the methanol-ether solution) by the method of Fiske & Subbarow (1925). After correction had been made for the various dilutions, the monoaminophospholipid was calculated on the basis of

$$\text{monoaminophospholipid} = \text{total P of trichloroacetic acid filtrate} \times 25.$$

An estimate of the lecithin was obtained by determining the choline in the hydrolysate by what was essentially the method of Glick (1944). To the remainder of the hydrolysate (equivalent to 21.7 ml. of the methanol-ether solution) there was added 1 drop of 1% (w/v) thymolphthalein in ethanol, followed by glacial acetic acid, drop by drop, until the blue colour disappeared. The solution was filtered through a Whatman no. 44 filter paper, and washed with three 2 ml. portions of distilled water.

To the filtrate was added 2 ml. 2% (w/v) ammonium reineckate in methanol, and the tube kept in the refrigerator overnight for the precipitation of the choline. The remainder of the procedure was carried out as described by Glick (1944), except that the choline reineckate was dissolved in a final volume of 4 ml. acetone, and the colour density measured in a Coleman Universal spectrophotometer at 526 $m\mu$. A standard solution containing 500 μ g. choline chloride was taken through at the same time. After correction had been made for the various dilutions, the lecithin was calculated on the basis of

$$\text{lecithin} = \text{choline (as choline chloride) in the trichloroacetic acid filtrate} \times 5.56.$$

The sphingomyelin and kephalin were calculated as follows

$$\begin{aligned} \text{sphingomyelin} &= \text{total phospholipid} \\ &\quad - \text{monoaminophospholipid} \\ \text{kephalin} &= \text{monoaminophospholipid} - \text{lecithin} \end{aligned}$$

RESULTS

The concentration of cerebroside, cholesterol and phospholipid in guinea pig, cat and rabbit brain is given in Table 1, and Table 2 gives the concentration of the individual phospholipids, lecithin, sphingomyelin and kephalin. In the experiments reported in these two tables the investigations were done on whole brain, no attempt being made to separate grey matter from white matter. It will be seen that the distribution pattern of the lipids was similar for each species studied.

Table 3 shows the cerebroside, cholesterol and phospholipid concentration in both grey matter and white matter from brains of the cat, dog, beaver and man, while Table 4 shows the concentration of each of the individual phospholipid fractions which constitute the total phospholipids.

The figures of Tables 1–4 are in terms of mg./100 mg. fresh tissue. It is seen that, on this basis, there was a greater concentration of cerebroside, total cholesterol, and total phospholipid in the white matter than in the grey for every species studied

Table 1. *Lipids in normal brain, expressed as mg./100 mg. fresh tissue (mean $\pm$ S.E. mean)*

	No. of animals	Cerebroside	Free cholesterol	Total cholesterol	Ester cholesterol	Total phospholipids
Guinea pig	8	2.29 $\pm$ 0.13	1.61 $\pm$ 0.09	1.76 $\pm$ 0.10	0.15 $\pm$ 0.07	4.82 $\pm$ 0.28
Cat	8	1.88 $\pm$ 0.12	1.89 $\pm$ 0.07	1.94 $\pm$ 0.07	0.05 $\pm$ 0.02	4.57 $\pm$ 0.29
Rabbit	4	2.74 $\pm$ 0.14	2.16 $\pm$ 0.06	2.21 $\pm$ 0.06	0.05 $\pm$ 0.02	5.40 $\pm$ 0.18

Table 2. *Phospholipids in normal brain, expressed as mg./100 mg. fresh tissue (mean $\pm$ S.E. mean)*

	No. of animals	Total phospholipids	Monoamino-phospholipids	Lecithin	Sphingomyelin (total phospholipid less monoamino-phospholipid)	Kephalin (monoamino-phospholipids less lecithin)
Guinea pig	8	4.82 $\pm$ 0.28	3.79 $\pm$ 0.13	—	0.94 $\pm$ 0.09	—
Cat	8	4.57 $\pm$ 0.29	3.69 $\pm$ 0.23	1.18 $\pm$ 0.08	0.93 $\pm$ 0.10	2.15 $\pm$ 0.35
Rabbit	4	5.40 $\pm$ 0.18	4.32 $\pm$ 0.16	1.39 $\pm$ 0.08	1.08 $\pm$ 0.10	2.92 $\pm$ 0.10

Table 3. *Lipids in grey matter and white matter of normal brain, expressed as mg./100 mg. fresh tissue*

	Cerebroside	Free cholesterol	Total cholesterol	Ester cholesterol	Total phospholipid	Total 'essential lipids'
Cat 1: Grey matter	1.10	1.24	1.29	0.05	4.54	6.93
White matter	4.83	4.42	4.42	0.00	8.18	17.43
Cat 2: Grey matter	1.86	1.19	1.20	0.01	4.03	7.09
White matter	5.37	4.64	4.64	0.00	7.73	17.74
Dog 1: Grey matter	1.49	1.29	1.36	0.07	4.20	7.05
White matter	6.79	5.39	5.39	0.00	8.95	21.13
Dog 2: Grey matter	1.54	1.29	1.30	0.01	4.19	7.03
White matter	7.42	4.86	4.86	0.00	7.99	20.27
Beaver: Grey matter	0.68	1.16	1.18	0.02	3.70	5.56
White matter	4.85	4.16	4.16	0.00	7.22	16.23
Human 1: Grey matter	1.20	1.00	1.00	0.00	3.12	5.32
White matter	4.61	3.69	4.00	0.31	6.24	14.85
Human 2: Grey matter	0.63	0.96	0.97	0.01	3.48	5.08
White matter	4.14	3.83	3.84	0.01	6.80	14.78

Table 4. *Phospholipids in grey matter and white matter of normal brain, expressed as mg./100 mg. fresh tissue*

	Total phospholipids	Monoamino-phospholipids	Lecithin	Sphingomyelin (total phospholipid less monoamino-phospholipids)	Kephalin (monoamino-phospholipids less lecithin)
Cat 1: Grey matter	4.54	3.86	—	0.68	—
White matter	8.18	6.02	—	2.16	—
Cat 2: Grey matter	4.03	3.29	1.35	0.74	1.94
White matter	7.73	3.95	1.36	3.78	2.59
Dog 1: Grey matter	4.20	3.26	0.77	0.94	2.49
White matter	8.95	4.66	1.65	4.29	3.01
Dog 2: Grey matter	4.19	3.28	1.23	0.91	2.05
White matter	7.99	4.95	1.62	3.04	3.33
Beaver: Grey matter	3.70	2.90	0.69	0.80	2.21
White matter	7.22	4.54	1.13	2.68	3.41
Human 1: Grey matter	3.12	2.82	0.61	0.30	2.21
White matter	6.24	3.64	0.90	2.60	2.74
Human 2: Grey matter	3.48	2.93	1.16	0.55	1.77
White matter	6.80	4.99	1.49	1.80	3.50

(Table 3). Each of the individual phospholipids (lecithin, sphingomyelin and kephalin) was present in a greater concentration in the white matter than in grey, but by far the greatest difference was in the sphingomyelin fraction (Table 4).

In Table 5 the various lipid fractions have been expressed as a percentage of the 'essential lipid', i.e. as a percentage of the sum of the cerebroside, total cholesterol and total phospholipid. On this basis it

DISCUSSION

The methods used allow triplicate estimations of all the substances measured to be done on samples of tissue as small as 500 mg. If single determinations only are done, samples weighing as little as 100 mg. may be used.

The extraction procedure and the published methods for the estimation of both free and total

Table 5. *Distribution of lipids in normal brain, expressed as percentage of 'essential lipids'*

	Cere- broside	Total chole- sterol	Total phospho- lipid	Lecithin	Sphingo- myelin	Kephalin
Cat 1: Grey matter	15.8	18.6	65.6	—	9.8	—
White matter	27.7	25.3	47.0	—	22.4	—
Cat 2: Grey matter	26.3	16.9	56.8	18.8	10.5	27.5
White matter	30.3	26.1	43.6	7.6	21.4	14.6
Dog 1: Grey matter	21.4	19.2	59.4	10.7	13.6	35.1
White matter	32.1	25.4	42.5	7.7	20.2	14.6
Dog 2: Grey matter	21.9	18.4	59.7	17.4	13.1	29.2
White matter	36.6	24.0	39.4	8.0	15.0	16.4
Beaver: Grey matter	12.3	21.4	66.3	12.4	14.3	39.6
White matter	29.9	25.8	44.3	6.9	16.6	20.8
Human 1: Grey matter	22.6	18.8	58.6	11.4	5.7	41.5
White matter	31.1	26.9	42.0	6.0	17.5	18.5
Human 2: Grey matter	12.4	19.1	68.5	22.7	10.8	35.0
White matter	28.0	26.0	46.0	10.1	12.2	23.7

is seen that, compared with grey matter, white matter contained relatively more cerebroside and cholesterol and less phospholipid. Of the individual phospholipids, however, white matter contained relatively more sphingomyelin and less lecithin and kephalin; thus, even although the concentration of total phospholipid in white matter was less on an 'essential lipid' basis, the concentration of sphingomyelin was greater. It follows, therefore, that the percentage of sphingomyelin in the total phospholipid fraction must be greater in white matter than in grey. This was observed to be so (Table 6).

Table 6. *Distribution of phospholipids in normal brain, expressed as percentage of total phospholipids*

	Lecithin	Sphingo- myelin	Kephalin
Cat 1: Grey matter	—	14.9	—
White matter	—	47.6	—
Cat 2: Grey matter	33.4	18.2	48.4
White matter	18.4	48.0	33.6
Dog 1: Grey matter	18.3	22.4	59.3
White matter	18.6	47.4	34.0
Dog 2: Grey matter	29.2	21.8	49.0
White matter	20.3	38.1	41.6
Beaver: Grey matter	18.6	21.6	59.8
White matter	15.7	37.1	47.2
Human 1: Grey matter	19.5	9.7	70.8
White matter	14.3	41.7	44.0
Human 2: Grey matter	33.2	15.8	51.0
White matter	21.9	26.6	51.5

cholesterol and of cerebroside proved satisfactory. At first sphingomyelin was measured by the reineckate method developed by Thannhauser and his colleagues (Thannhauser & Setz, 1936*a, b*; Thannhauser & Benotti, 1938). This method, combined with the estimation of the choline-containing phospholipids, was used for determining the phospholipid distribution in tissues as described by Thannhauser, Benotti & Reinstein (1939), and adapted for small samples of tissue by Erickson, Avrin, Teague & Williams (1940). The reineckate method proved unsatisfactory in our hands, however, and it is interesting to note that it has also been criticized by Hack (1946), Schmidt *et al.* (1946) and Thannhauser, Benotti & Boncoddio (1946).

There are few reports in the literature on the distribution of the lipids in the brain of the guinea pig, rabbit and cat, and, as far as we know, none on that of the beaver. The figures presented here are comparable to those reported for dog brain by Erickson *et al.* (1940), for ox brain by Kaucher, Galbraith, Button & Williams (1943) and for rat brain by Williams, Galbraith, Kaucher, Moyer, Allen & Macy (1945).

The finding that there was a greater concentration of cerebroside, cholesterol and sphingomyelin in white matter than in grey matter is in agreement with the work of Randall (1938) and Yasuda (1937), who found that there was a greater concentration of cholesterol and phospholipid in white matter than

in grey. The greater concentration of cholesterol in white matter has also been observed by Petrowsky (1873), Thudichum (1901) and Kirschbaum & Linnert (1912), and the greater concentration of cerebroside by Petrowsky (1873) and Smith & Mair (1912-13).

In the present study it was shown that, on a wet weight basis, there was a greater concentration of all three phospholipids—lecithin, sphingomyelin, and kephalin—in the white matter than in the grey, but that the increase was chiefly in the sphingomyelin fraction. This is in confirmation of the observation of Schmidt *et al.* (1946), who found that the concentration, on a wet weight basis, of sphingomyelin in the white matter of ox brain was much greater than that in the grey matter.

The figures reported for the relative concentrations of lecithin, sphingomyelin and kephalin for grey matter are similar to those obtained for whole brain by other workers using the method of Thannhauser, Benotti & Reinstein (1939). For instance, Thannhauser, Benotti, Walcott & Reinstein (1939) for human brain, Erickson *et al.* (1940) for dog brain, Kaucher *et al.* (1943) for ox brain, and Williams *et al.* (1945) for rat brain found that the concentration of sphingomyelin was roughly the same as that of lecithin, and that the concentration of kephalin was much greater. In white matter the concentration of sphingomyelin relative to that of lecithin was found to be much greater than in grey, whereas the concentration of kephalin relative to that of lecithin was not greatly different.

White matter is thus seen to be characterized by a high concentration of 'essential lipid', there being relatively more cerebroside, cholesterol and sphingo-

myelin than is present in grey matter, with little difference in the case of lecithin or kephalin. This was found to be true for all the species studied, the cat, dog, beaver and man, and it is tempting to speculate that it is the cerebroside, cholesterol and sphingomyelin, rather than lecithin and kephalin, that form the basis of 'myelin'.

SUMMARY

1. The concentration of cerebroside, free cholesterol, total cholesterol, total phospholipid, lecithin, sphingomyelin and kephalin has been determined in the whole brains of a series of guinea pigs, cats and rabbits.

2. Similar studies have been made on both the white matter and grey matter from brains of the cat, dog, beaver and man.

3. On a wet weight basis, there was a greater concentration of cerebroside, total cholesterol, total phospholipid, lecithin, sphingomyelin and kephalin in white matter than in grey matter.

4. Referred to total 'essential lipid' the concentration of cerebroside and total cholesterol was greater in white matter than in grey, while the concentration of total phospholipid was less. However, the concentration of sphingomyelin was greater in white matter, this being offset by a decrease in the concentration of both lecithin and kephalin.

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